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(21) International Application Number: PCT/US94/14502 (22) International Filing Date: 14 December 1994 (14.12.94) (30) Priority Data: 08/166,925 14 December 1993 (14.12.93) US (60) Parent Application or Grant (63) Related by Continuation US 08/166,925 (CIP) Filed on 14 December 1993 (14.12.93) (71) Applicant (for all designated States except US): CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): FALCK-PEDERSEN, Erik S. [US/US]; 1161 York Avenue, New York, NY 10021 (US). (74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: ADENOVIRUS GENE EXPRESSION SYSTEM (57) Abstract The present invention provides a novel, highly efficient, recombinant adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus was produced by cotransfecting a novel vector with the large fragment of the adenovirus-5 genome in 293 cells. Homologous recombination between these two DNA fragments resulted in the production of the recombinant adenovirus expression system. This vector, when converted to a recombinant virus has the unique capability of expressing one or more heterologous genes at very high levels. The novel vector, comprises, at least one cDNA insertion site for cloning a selected heterologous gene; a promoter sequence positioned upstream from the gene insertion site; the left end replication and packaging elements of the adenovirus-5 genome positioned upstream of the promoter; a highly efficient eukaryotic splice acceptor and splice donor site positioned immediately downstream of the promoter; and positioned downstream of the insertion site a strong polyadenylation sequence and the region for homologous recombination containing a portion of the adenovirus-5 genome. Between the packaging sequence and the CMV promoter are restriction sites for insertion of a second fully functional transcription unit.		

FIELD OF THE INVENTION

10 BACKGROUND OF THE INVENTION

E1 and E3 and a site upstream of E4 have been utilized as sites for insertion of foreign DNA sequences in the generation of recombinant adenoviruses (Berkner et al.,

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Adenovirus expression vectors have been in use for the past decade (Thummel et al., 1981, *Cell*, vol. 23, pp. 825-836; Berkner et al., 1984, *Nucleic Acids Res.*, vol. 12, pp. 1925-1941; and for a review see Grunhaus et al., 1992, *Seminars in Virology* 3, pp. 237-252), and more recently exploited for the purpose of gene therapy (Herz et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 90, pp. 2812-2816; Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434; and Rosenfeld et al., 1992, *Cell*, vol. 68, pp. 143-155). Features of adenovirus based expression vectors which make them attractive to gene therapy applications include very efficient uptake into cells which contain the appropriate adenovirus receptor and uptake pathway, and the ability to carry up to 7.5 kb of foreign DNA. Adenovirus vectors allow a reporter gene to be under the control of tissue specific promoter elements (Friedman et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3791-3797; and Babiss et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3798-3806) as well as a variety of viral and mammalian constitutive promoter elements (Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90).

One such example of an adenovirus-based vector system is described in Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90. The authors here describe a helper-independent adenovirus type 5-luciferase recombinant containing the firefly luciferase gene flanked by simian virus 40 (SV40) regulatory sequences inserted into the early region 3 (E3) of the adenovirus-5 genome. A plasmid containing the luciferase gene and SV40 regulatory sequences in the E3 region was co-transfected with a plasmid containing the adenovirus-5 d1309 genome in circular form. Upon transfection of 293 cells, virus progeny produced by *in vivo* recombination between the two plasmids resulted in rescue of the adenovirus type 5-luciferase recombinant (i.e., E3 insert in Adenovirus-5 genome).

Gomez-Foix et al., 1992, *J. Biol. Chemistry*, vol. 267, no. 35, pp. 25129-25134, discloses adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes in culture. The preparation of a recombinant adenovirus containing the cDNA encoding rabbit muscle glycogen phosphorylase is described whereby the cytomegalovirus (CMV) early gene promoter/enhancer, pUC 18 polylinker, fragment of the SV40 genome that includes the small T-antigen intron and the polyadenylation

Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434, discloses adenovirus-mediated transfer of recombinant α 1-antitrypsin gene to the lung epithelium cells of the cotton rat respiratory tract *in vivo*. The adenoviral vector contained an adenovirus major late promoter and a recombinant human α 1-antitrypsin gene.

- 5 Quantin et al., 1992, *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 2581-2584, discloses a recombinant adenovirus containing the β -galactosidase reporter gene under the control of muscle-specific regulatory sequences. This recombinant virus directed expression of the β -galactosidase in myotubes *in vivo*.

- Problems associated with adenovirus infection, particularly those associated with
10 repression of host cell mRNA translation and shutdown of host normal mRNA production (Babich et al., 1983, *Mol. Cell. Biol.*, vol. 3, pp. 1212-1221; Beltz et al., 1979, *J. Mol. Biol.*, vol. 131, pp. 353-373; Schneider et al., 1987, *Annu. Rev. Biochem.*, vol. 56, pp. 317-332) have been addressed by using defective adenovirus vectors which are based on mutations in the dominant regulatory region, E1 (Harrison
15 et al., 1977, *Virology*, vol. 77, pp. 319-329; Jones et al., 1979, *Cell*, vol. 17, pp. 583-689). In addition, conventional adenovirus vector systems typically require high cell exposure (e.g., MOI's in excess of 500 PFU/cell) for expression of the desired gene, which is detrimental to the cells because of cytopathic effects from exposure. Therefore, a need exists for an adenovirus-mediated expression vector which can infect
20 cells at low doses, yet can exhibit maximum expression of a gene in the cell.

- Moreover, although adenovirus-based vectors for gene expression have been successfully employed with a number of mammalian and viral genes (for review, see Mulligan, R.C., 1993, *Science*, vol. 260, pp. 926-932), they have not apparently been used to express any member of the guanine nucleotide-binding protein coupled
25 receptors (GPCR) family, such as the pituitary thyrotropin-releasing hormone (TRH-R) (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp. 9514-9518; Yamada et al., 1992, *Biochem. Biophys. Res. Commun.*, vol. 184, pp. 367-372; Zhao et al., 1992, *Endocrinology*, vol. 130, pp. 3529-3536; de la Pena et al., 1992, *Biochem. J.*, vol. 284, pp. 891-899). Seven transmembrane-spanning GPCRs comprise a large
30 family of cell surface regulatory proteins (Dohlman et al., 1991, *Annu. Rev. Biochem.*,

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which have successfully taken up sufficient quantities of DNA (Kitsis et al., 1991, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 88, pp. 4138-4142; Lin et al., 1990, *Circulation*, vol. 82, pp. 2217-2221; and Ascadi et al., 1991, *The New Biologist*, vol. 3, pp. 71-81). The problem associated with direct DNA injection is its relative inefficiency as only
5 approximately 0.02% of the myocytes in the adult rat heart take up and express injected DNA (Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K. W., Academic Press, Inc., New York, Vol. 1, pp. 374-392).

A recent report demonstrated efficient gene transfer into adult rat cardiocytes *in vitro* (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387). In addition,
10 recent studies using adenovirus vectors introduced intravenously into both rats and mice, indicate that the virus will infect a wide variety of tissue types, including mouse skeletal and cardiac muscle (Quantin et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 89, pp. 2581-2584; and Stratford-Perricaudet et al., 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630). However, little quantitative data is available concerning expression of
15 adenovirus-mediated gene transfer *in vivo*. Therefore, a need exists for an adenovirus-mediated gene transfer vector system which would function effectively with primary cultures of cardiac myocytes and one which would also have application *in vitro*.

SUMMARY OF THE INVENTION

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The primary object of the present invention is to provide an adenovirus-based expression system capable of expressing a heterologous gene(s) in a host mammalian cell.

The present invention provides a novel, highly efficient, recombinant
25 adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus expression system of the invention was produced via homologous recombination between the novel vector of the invention co-transfected with the large fragment of the adenovirus-5 genome in 293 cells.

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derivative thereof. Homologous recombination between these DNA fragments results in the production of a replication defective, recombinant adenovirus. The recombination reconstructs the adenovirus-5 genome by displacing the E1A and E1B protein coding regions with the plasmid vector cDNA.

5 In another embodiment of the invention, there is provided a recombinant adenovirus expression system for the receptor for thyrotropin-releasing hormone (TRH-R). The recombinant adenovirus, designated AdCMVmTRHR, circumvents difficulties encountered when using conventional transient or stable plasmid expression systems. Using this recombinant adenovirus (AdCMVmTRHR), TRH-Rs can be expressed in
10 different mammalian cell types, including those resistant to transient transfection assay. Recombinant adenovirus, AdCMVmTRHR, was produced by homologous recombination between plasmid vector, designated pAdCMVmTRHR, i.e. the generic plasmid vector of the invention containing the gene coding TRH-R, co-transfected with the large fragment of adenovirus-5 d1309 genome. The versatility of using adenovirus
15 mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology, but provides a valuable *in vivo* expression vector capable of extending TRH-R studies in animal model systems.

In a further embodiment of the present invention, infection of cultured fetal and adult rat cardiac myocytes *in vitro* and of adult cardiac myocytes *in vivo* was
20 characterized using the recombinant adenovirus of the invention. The recombinant adenovirus, designated AdCMVCATgD, includes the chloramphenicol acetyltransferase (CAT) reporter gene driven by the cytomegalovirus (CMV) promoter. Plasmid vector pAdCMVCATgD i.e., generic plasmid vector of the present invention containing the gene encoding the bacterial CAT sequence, was co-transfected with the large fragment
25 of the adenovirus-5 genome (3.6-100 map units). Homologous recombination between the plasmid vector and adenovirus fragment produced the recombinant adenovirus, designated AdCMVCATgD.

Virtually all fetal or adult cardiocytes expressed the CAT gene *in vitro* when infected with 1 plaque forming unit (pfu) of virus per cell. Using *in vitro* studies as a
30 guide, recombinant virus AdCMVCATgD was introduced directly into adult rat

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Fig. 12 is a graphic map showing the structure of plasmid vector pML-E1aEF-5778.

Fig. 13 is a graphic map of plasmid vector pAdCMVmTRHR used for the construction of recombinant adenovirus AdCMVmTRHR.

5 Fig. 14 is a graph showing a comparison of infection with AdCMVmTRHR and transfection with pAdCMVmTRHR on expression of TRH-Rs and *methyl*/TRH responsiveness in six mammalian cell lines.

Fig. 15 is a graph showing TRH-induced desensitization and PMA-induced inhibition of the TRH response in AdCMVmTRHR-infected GHY, COS-1 and KB
10 cells.

Fig. 16 is a graph showing *methyl*/TRH-stimulated TRH-R internalization in AdCMVmTRHR-infected GHY, COS-1 and KB cells.

Fig. 17 is a graphic map of plasmid vector pAdCMVCATgD used in construction of recombinant adenovirus AdCMVCATgD.

15 Fig. 18(a) is a graph showing dosage and time dependent expression of adenovirus in fetal cardiocytes.

Fig. 18(b) is a graph showing dosage and time dependent CAT expression following infection by AdCMVCATgD in adult cardiocytes.

Fig. 19 is a graph showing distribution of CAT activity in cells of
20 AdCMVCATgD injected hearts.

Fig. 20A is a graph showing CAT expression in the left ventricle 5 days following intracardiac injection of four doses of adenovirus [AdCMVCATgD; 6×10^6 , (n=4); 6×10^7 , (n=4); 6×10^8 , (n=3); and 2×10^9 , (n=2)].

Fig. 20B is a graph showing CAT expression over time in the left ventricle
25 following injection of 6×10^7 pfu of AdCMVCATgD virus.

Fig. 21 (a-f) is an immunohistochemical staining for CAT protein in adenovirus infected hearts.

Fig. 22 is a schematic showing the nucleotide sequence of plasmid vector pAdCMV-HS-Vector, as shown in Figs. 1(a) & 1(b).

30 Fig. 23 is a schematic showing the nucleotide sequence of another version of plasmid vector pAdCMV-HS-vector.

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electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. These procedures are generally well known. For example, see Lawn et al., 1981, *Nucleic Acids Res.*, vol. 9, pp. 6103-6114; and Goeddel et al., 1980, *Nucleic Acids Res.*, vol. 8, p. 4057, which disclosures are hereby incorporated by reference.

The term "expression" may be characterized as follows: A cell is capable of synthesizing many proteins. At any given time, many proteins which the cell is capable of synthesizing are not being synthesized. When a particular polypeptide, coded for by a given gene, is being synthesized by the cell, that gene is said to be expressed. In order to be expressed, the DNA sequence coding for that particular polypeptide must be properly located with respect to the control region of the gene. The function of the control region is to permit the expression of the gene under its control.

The term "southern blot analysis" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically comprises electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane supports for analysis with a radiolabeled, biotinylated or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

The term "northern analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ³²P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or poly-acrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art, such as those described in sections

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compatible for ligation. To blunt the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15° C, with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenolchloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 g of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase, or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large and small scale plasmid preparations described in sections 1.25-1.33 of Sambrook et al., supra, which disclosure is hereby incorporated by reference. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

This invention achieves significantly enhanced *in vitro* and *in vivo* expression levels of heterologous gene(s) by inserting into a host mammalian cell the adenovirus expression system or plasmid vector of the invention, containing foreign cDNA encoding the heterologous gene(s) under the transcriptional control of DNA fragments derived from the mouse cytomegalovirus (CMV) immediate early gene regulatory sequences. It is understood that the CMV immediate early promoter can be combined with enhancer elements isolated from other transcriptional units to increase expression efficiency.

The recombinant adenovirus expression system and plasmid vector include at least one cDNA insertion site(s) i.e., restriction site(s) for cloning a selected heterologous gene(s). Other important features of the adenovirus expression system and plasmid vector of the invention include a highly efficient eukaryotic splicing

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nucleotide sequence from nucleotide 2800-5776, which serves as the region for homologous recombination.

To obtain efficient expression of the heterologous gene(s), a eukaryotic promoter must be present in the plasmid vector and recombinant adenovirus expression system. It is understood that any known eukaryotic promoter can be utilized in the plasmid vector and/or recombinant adenovirus expression system of the invention provided the promoter is capable of expressing the heterologous gene(s). The promoter used herein, preferably, is the mouse cytomegalovirus-1 early promoter, or an effective expression promoting fragment thereof. For an example of the CMV promoter, see U.S. Patent No. 4,963,481 to Jean P. deVilliers, which disclosure is hereby incorporated by reference. The use of the mouse CMV promoter is of broad utility because this promoter has a very broad host range and functions with superior strength and efficiency in a wide variety of cell lines tested.

The presence and position of the splicing elements with respect to the cDNA are important to overall processing efficiency, as is the choice of splicing elements. In the present invention, a hybrid splice donor and acceptor was used which yielded a highly efficient processing activity compared to the more common splice element used in other systems i.e., the SV40 small T splice site. By inserting the cDNA downstream of the splice elements, we are coupling the splice elements to the downstream 3' processing site generating a terminal exon. Use of a demonstrably efficient poly(A) site maximizes efficiency of the expression system. This allows efficient conversion of pre-mRNA into mRNA and allows the system to take full advantage of the high level of expression generated by the CMV promoter.

Any of the conventional cloning methods for insertion of the gene and/or gene fragment(s) into the plasmid vector can be used to ligate the promoter and the other control elements into specific sites within the plasmid vector. Accordingly, heterologous gene sequence(s) containing those regions coding for the gene(s) can be ligated into the plasmid vector at a specific restriction site in relation to the promoter and control elements so that when either the recombinant adenovirus or plasmid vector

chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard techniques.

The protein(s) encoded by the heterologous gene(s) inserted into the plasmid vector and recombinant adenovirus expression system can comprise any known protein, including; growth hormone, human growth hormone (HGH), des-N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin B-chain, proinsulin, relaxin A-chain, relaxin B-chain, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), leutinizing hormone (LH), glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, human tissue-type plasminogen activator (t-PA), bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor- α and - β , enkephalinase, human serum albumin, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, β -lactamase, tissue factor protein, inhibin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF- β platelet-growth factor, transforming growth factor; TGF- α and TGF- β insulin-like growth factor-I and -II, insulin-like growth factor binding proteins, CD-4, DNase, latency associated peptide erythropoietin, osteoinductive factors, interferon, α , - β , and - γ , colony stimulating factors (CSFs), M-CSF, GM-CSF, and G-CSF, interleukins (ILs), IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, superoxide dismutase; viral antigens; HIV envelope proteins GP120 and GP140, immuno globulins, and fragments of the above listed proteins.

The following Examples are provided to further illustrate the present invention.

Example I

Construction of pGEM2AdCMV

Plasmid pBstSK+ (0-353) contains adenovirus-5 sequence from nt 0-353 inserted into pBstSK+ vector at the *EcoRI* site and the *SsrII* site (which has been lost by blunt end ligation). These sequences are required viral elements which include the origin for DNA replication and the viral packaging sequence. The CMV

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Example IV**Construction of pAdCMVdHCatgD**

One of the three *Hind*III restriction sites in pAdCMVCatgD (Figure 6) was
5 deleted by partial *Hind*III digestion and filling by Klenow large fragment of DNA
polymerase followed by plasmid circularization and ligation. This allowed removal of
the CAT sequence and the poly(A) site by *Hind*III digestion, with the retention of
promoter and splicing sequences. A 1100 bp of E1B sequence was deleted.

10

Example V**Construction of pAdCMVCatgDNeo(-)**

The unique restriction site *Nor*I located at position 361 can be used to insert any
additional gene of interest. As a test construct a *Nor*I fragment from pPYNeo was
15 isolated which contained the Neomycin resistance gene driven by the polyoma
promoter and using the SV40 splicing and polyadenylation elements. This strategy
resulted in the introduction of the Neo gene into the vector in two orientations relative
to the direction of CAT gene expression (+) and (-). Both of these constructs were
used in virus constructions, however, only the AdCMVCatDNeo(-) virus has been
20 isolated to date.

Example VI**Construction of pAdCMVTRHrE3**

25 Using vector pAdCMVdHCatgD (Figure 6), cDNA for thyrotropin releasing
hormone receptor (which contains the adenovirus E2 poly(A) site) was inserted
directly into the *Hind*III digested vector to construct vector pAdCMVTRHrE3
(Figure 9).

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2. Construction of AdCMVmTRHR:

The parent plasmid, pAdCMVmTRHR, was constructed by inserting a 1.2 kb *EcoRI-NorI* fragment containing the protein-coding region of the mouse TRH-R cDNA, nucleotides 233-1462 of plasmid pBSmTRHR (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp. 9514-9518, which disclosure is hereby incorporated by reference), into plasmid pGEM2-L3-114 at the *EcoRI-BamHI* site. After digesting with *EcoRI* and using the Klenow fragment of DNA polymerase I to make blunt DNA ends, *HindIII* linkers were ligated and a 1.4 kb *HindIII* fragment containing mouse TRH-R cDNA and the adenovirus E2 poly(A)-signal sequence was isolated and inserted into the *HindIII* site of the pAdCMV-HS-Vector (i.e., expression plasmid of the present invention) which contains the left end replication and packaging elements of adenovirus, the cytomegalovirus-1 promoter and splicing elements from plasmid pML-IS Cat (Huang et al., 1990, *Nucleic Acids Res.*, vol. 18, pp. 937-947, which disclosure is hereby incorporated by reference). Following verification of the plasmid by restriction site mapping and transient transfection of pAdCMVmTRHR into COS-1 cells to demonstrate TRH-R expression, the virus AdCMVmTRHR was constructed by overlap recombination as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. All transfections were carried out in human embryonic kidney cells transformed with the E1 region of adenovirus type 5 according to the procedure of Graham et al., 1977, *J. Gen. Virol.*, vol. 36, pp. 59-72, which disclosure is hereby incorporated by reference. Following plaque purification, virus was grown in 293 cells in suspension cultures as described by Antravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. The entire sequence coding for the adenovirus E1a gene was removed as well as the 5' 1.8 kb of the E1b gene. Co-transfection of pAdCMVmTRHR with the large fragment of adenovirus (3.8-100 map units) into 293 cells resulted in production of recombinant virus AdCMVmTRHR.

9514-9518, which disclosure is hereby incorporated by reference). For GHY, AtT-20 and C6 cells, incubation with plasmid and DEAE dextran was for 0.5 hours at 4°C and no chloroquine or dimethylsulfoxide was added (Fujimoto et al., *Endocrinology*, vol. 130, pp. 1879-1884, which disclosure is hereby incorporated by reference). Cells were studied 48 to 72 hours after transfection, which are times of maximum TRH-R expression.

5. Measurement of TRH-R number:

Binding of 0.1 to 7.5 nM [³H]methy/TRH, an analog of higher affinity and potency than TRH (Vale et al., 1971, *Endocrinology*, vol. 89, pp. 1485-1488, which disclosure is hereby incorporated reference), to intact cells was measured as described by Gershengorn, M.C., 1978, *J.Clin. Invest.*, vol. 62, pp. 937-943, which disclosure is hereby incorporated by reference. Binding isotherms were fitted and dissociation constants (K_d s) and receptor numbers (one-to-one stoichiometry of methy/TRH and receptor) were obtained with the INPLOT program (Graphpad). Receptor number was calculated using the following equation: fractional occupancy = $1 / [1 + (K_d/L)]$. Receptor number is given assuming that all cells in the population are expressing equal numbers of TRH-Rs. This appears to be the case with infections using 300 AdCMVmTRHR particles per cell (not shown).

6. Measurement of TRH response:

Infected or transfected cells were labelled for 24 hours with [³H]myo-inositol, stimulated with TRH or methy/TRH in a balanced salt solution containing 10 mM LiCl and [³H]IPs were measured as described by Imai et al., 1987, *Methods. Enzymol.*, vol. 141, pp. 100-101, which disclosure is hereby incorporated by reference.

7. Measurement of desensitization and inhibition by PMA:

Cells were incubated in medium with serum containing myo-[³H]inositol (1 μ Ci/ml) for 24 hours prior to infection and studied 16 to 24 hours after infection.

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AdCMVmTRHR (Fig. 13) was similar to that used in the construction of the plasmid vector constructs of the invention (Figs. 1(a) & 1(b)). With reference to Figure 13, there is shown plasmid vector pAdCMVmTRHR, which was used to produce recombinant adenovirus AdCMVmTRHR. Turning to Figure 13, the left end of the adenovirus starts at position 1. The adenovirus sequence from nucleotide 1-353 contains the origin of replication and the viral packaging sequence. The adenovirus sequence from 354-2800 is deleted and replaced with the CMV-1 promoter, splice elements, the protein coding region of the mouse TRH-R cDNA sequence and the E2 poly(A) site. The left end adenovirus sequence from nucleotides 2800-5776 serve as the region for homologous recombination.

The novelty of AdCMVmTRHR as a vector for expression of TRH-Rs and its advantage over transfection are illustrated in Figure 14. With reference to Figure 14, the levels of TRH-R expression (upper panel) and *methy*/TRH stimulation of [³H]IP formation (lower panel) were measured as previously described. The data in the (upper panel) are presented as number of receptors per cell assuming that all cells express equal numbers of TRH-Rs. The bars in both panels represent the mean \pm SD of triplicate determinations in a representative experiment that was performed 3 times. In these experiments the plasmid vector used for virus construction, and expression of TRH-Rs after infection with AdCMVmTRHR and after transfection with pAdCMVmTRHR, were compared in HeLa cells, rat pituitary tumor GHY cells, mouse pituitary tumor AtT-20 cells, rat glioma C6 cells and monkey kidney CV1 and COS-1 cells. These cell lines were chosen because they represent a wide variety of cell types which do not express TRH-Rs. That is, HeLa cells were studied because they are readily infected with adenovirus. GHY cells were studied because they are a subclone of the cells in which endogenous TRH-Rs have been most well-studied. COS-1 cells were studied because they are a commonly used, transformed cell line that permits high levels of expression during transient assays.

TRH-Rs expressed on the surface of these cells after infection with AdCMVmTRHR bound *methy*/TRH with the same affinity as native TRH-Rs on mouse pituitary cells (Gershengorn et al., 1978, *J. Clin. Invest.*, vol. 62, pp. 937-943, which

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all cell types after infection by AdCMVmTRHR than after transfection. However, there was no correlation between the magnitude of the *methy*lTRH response and the number of TRH-Rs when comparing different cell types. For example, *methy*lTRH stimulation of IP formation was greater in AtT-20 cells which expressed TRH-Rs at a lower number than in HeLa cells with a greater number of TRH-Rs. One explanation for this observation may be that there are differences in post-receptor components of the signal transduction cascades within these different cell types. Another finding was that the magnitude of response to *methy*lTRH in COS-1 cells was greater after infection than after transfection even though the total number of receptors was similar. This may be because all COS-1 cells expressed a maximally effective number of TRH-Rs after AdCMVmTRHR infection, whereas only a fraction of the transfected cells were expressing maximally effective numbers of TRH-Rs because infection is more efficient than transfection.

In rat GH₃ pituitary cells naturally expressing TRH-Rs, the TRH response is rapidly desensitized (Perlman et al., 1991, *Endocrinology*, vol. 129, pp. 2679-2686, which disclosure is hereby incorporated by reference). This effect occurs prior to any decrease in the number of TRH-Rs ("down-regulation") (Gershengorn, M. C., 1978, *J. Clin. Invest.*, vol. 62, pp. 937-943; and Hinkle et al., 1975, *Biochemistry*, vol. 14, pp. 3845-3851, which disclosures are hereby incorporated by reference. This response to TRH is also blunted in GH₃ cells preincubated with phorbol esters, such as PMA, which activate protein kinase C (Drummond, A. H., 1986, *J. Exp. Biol.*, vol. 124, pp. 337-358, which disclosure is hereby incorporated by reference). Evidence, however, is presented that these two effects are distinct and suggested that TRH-induced desensitization is not mediated primarily by protein kinase C (Perlman et al., 1991, *Endocrinology*, vol. 129, pp. 2679-2686, which disclosure is hereby incorporate by reference). Although the molecular mechanisms of TRH-induced desensitization and of PMA-induced inhibition of the TRH response have not been elucidated, it is likely that they are mediated by receptor phosphorylation (Lefkowitz et al., 1992, *Cold Spring Harbor Symp. Quant. Biol.*, vol. 57, pp. 127-134, which disclosure is hereby incorporate by reference). Because different cell types contain

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Rapid internalization is another process that many GPCRs, including TRH-Rs (Nussenzveig et al., 1993, *J. Biol. Chem.*, vol. 268, pp. 2389-2392; and Hinkle, P. M., 1989, *Ann. N. Y. Acad. Sci.*, vol. 553, pp. 176-187, which disclosures are hereby incorporated by reference), undergo after binding (Dohlman et al., 1991, *Annu. Rev. Biochem.*, vol. 60, pp. 653-688, which disclosure is hereby incorporated by reference).

To determine whether TRH-R internalization is cell type specific, we measured internalization of bound *methy*/TRH in three AdCMVvTRHR-infected cell lines which displayed differences in TRH-induced desensitization or PMA-induced inhibition of the TRH response, or both. Internalization in cell lines that do and do not exhibit rapid desensitization induced by TRH was measured because it has been controversial whether these two processes are related. Figure 16 illustrates that internalization of *methy*/TRH-bound TRH-Rs was faster in AdCMVmTRHR-infected GHY cells than in COS-1 cells and KB cells but that the fraction of receptors internalized after 60 minutes was similar in all three cell types. After 60 minutes, $64 \pm 7.0\%$, $62 \pm 2.1\%$, and $71 \pm 2.6\%$ of TRH-Rs were internalized in AdCMVmTRHR-infected GHY, COA-1 and KB cells, respectively. With reference to Figure 16, internalization of TRH-Rs was measured as previously described. The data represent mean \pm SD of triplicate determinations in a representative experiment performed twice. In these three cell lines, agonist-induced internalization of TRH-Rs exhibited small kinetic differences but the extent of internalization after 60 minutes, the time at which measured desensitization were similar.

A number of aspects of GPCR biology may vary when receptors are expressed in different cell types. For example, the same GPCR may activate different signal transduction pathways when expressed in different cell types (Milligan et al., 1993, *Trends Pharmacol. Sci.*, vol. 553, pp. 176-187, which disclosure is hereby incorporated by reference). Agonist-induced desensitization, which is a process that commonly accompanies activation of GPCRs, appears to be mediated by a conserved set of intracellular regulatory proteins including protein kinases and arrestin-like proteins (Lefkowitz et al. 1993, *Adv. Second Messenger Phosphoprotein Res.*, vol. 28, pp. 1-9; and Lefkowitz, R. J., 1993, *Cell*, vol. 74, pp. 409-412, which disclosures are hereby

Example VIII

Quantitative Determination of Adenovirus-Mediated Gene Delivery to Rat Cardiac Myocytes *In Vitro* and *In Vivo*

5 1. Isolation and culture of Rat Cardiac Myocytes:

Primary fetal cardiac myocytes were prepared from fetal day 20 Sprague-Dawley rats (Taconic Farms) by modification of the protocol of de Carvalho et al., 1992, *Circ. Res.*, vol. 70, pp. 733-742, which disclosure is hereby incorporated by reference. Cardiac cells were preplated for 1 hour in order to remove fibroblasts.

10 1.8X10⁶ cells were then plated per 25mm tissue culture dishes (Corning) in heart medium (Hank's salt solution supplemented with MEM Vitamin Stock, MEM amino acids, MEM non-essential amino acids, L-Glutamine (2mM), 1% Glycine, 2% Hypoxanthine, 1% Penn-Strep, NaHCO₃) with 10% fetal bovine serum (Hyclone). Primary adult cardiac myocytes were prepared from the hearts of 200g female

15 Sprague-Dawley rats (Taconic Farms) according to the protocol of White et al. 1993, *Biophys. J.*, vol. 65, pp. 196-204, which disclosure is hereby incorporated by reference. 2.4 x 10⁵ cells were plated in heart medium per 60mm dish coated with 20 ug/ml of laminin (Boehringer Mannheim). Cells were maintained in culture at 37°C, 5% CO₂. Cell culture medium was changed every other day for the duration of the assay.

20

2. Virus production:

Virus plating and the preparation of viral stocks were performed on 293 monolayer cells as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference.

25

3. Infection of cardiac myocytes:

Forty eight hours after plating, fetal myocytes were infected with AdCMVCATgD at 0.01, 0.1, 1, 10 pfu/cell. The adult cells were infected with the same doses immediately after plating. AdCMVCATgD (10¹⁰ pfu/ml) was diluted in

30 heart media without added serum. One ml of media + virus was added to each 60mm

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were then washed in dH₂O and mounted with gelvatol (Airvol, Air Products and Chemicals, Inc.).

5. CAT assays from myocytes:

5 At each time point, infected cardiac myocytes were harvested according to the protocol of Ausbel et al., 1989, *Current Protocols in Molecular Biology*, Wiley, New York, which disclosure is hereby incorporated by reference. The amount of protein in the supernatant was measured by Bradford assay using bovine serum albumin (BSA) as the standard (BioRad). CAT assays were performed on 10 µg of
10 total protein. When the amount of CAT activity was greater than 70% and out of the linear range, supernatants were diluted in 0.1 mg/ml BSA. CAT assays were done by TLC according to the method of Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc., New York, Vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference, incubating for 2 hours at 37°C.

15

6. DNA and virus injections in vivo:

10 µg of CMV CAT plasmid DNA in 50 µl PBS was injected into the apex of the left ventricle of 200g female Sprague Dawley rats as described by Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc.,
20 New York, vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference. For the adenovirus injections, 6×10^6 to 6×10^8 pfu in 50 µl PBS were injected, 2×10^9 pfu were injected undiluted in a volume of 50 µl.

7. CAT assays on tissue:

25 At indicated times following injection, hearts were removed, rinsed in PBS and weighed. For the spatial distribution experiment the hearts were then sectioned into seven roughly equivalent slices. Each slice was then homogenized using a Tissumizer (Tekmar) in a volume of 0.5 mls buffer (1M Gly gly pH 7.8, 150mM MgSO₄, 500mM EGTA pH 8.0, 1M DTT) for 20 sec. For the dosage, and time course
30 experiments the hearts were homogenized the same way but in a volume equal to 0.5g

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dilutions of cell extracts were made to maintain assays in the linear range of the CAT assay.

CAT activity was easily detected at the earliest measured time point (4 hours), was near maximal by 48 hours, and was maintained at stable levels through the remainder of the experiment (a total of 167 hours). A dose-dependent increase was maintained over a range of hour logs of virus input throughout much of the time course. The same basic extent and level of infection and expression was found in adult cardiocytes (Figure 16(b)) when infected under similar conditions. However, the duration of study was shortened to 48 hours due to the difficulty in maintaining healthy differentiated adult cardiac myocytes in culture, independent of virus infection. Based on these assays, the sensitivity of the AdCMVCATgD CAT assay, and the levels of activity resulting from these infections, it was redacted that CAT expression could be reliably detected in as few as 10 infected cells.

At each dose of virus, the percentage of fetal cells which were expressing CAT was determined by immunostaining coverslips of infected fetal cardiocytes 18 and 48 hours post infection. Mock-infected cells show no staining, but cells infected with increasing doses of virus show a proportional increase in the number of cells infected, with 1 pfu/cell (100 particles) resulting in virtually 100% of the cells being stained (data not shown). The virus infection included both myocytes and the small proportion of nonmyocyte fibroblasts (<5%) which remained in the culture following initial myocyte purification (data not shown). Similar results were obtained with adult cardiac myocytes. At an infection of 1 pfu or greater, 100% of the rod-shaped adult myocytes stained positive with an anti-CAT antibody. This was true at both 4 and 48 hours. Myocytes which were rounded up also stained positive for CAT, and sarcomeric myosin heavy chain, and excluded trypan blue (data not shown).

Adenovirus mediated gene transfer offers advantages to transient transfection assays when using cultured myocytes. The quantitative advantages of using AdCMVCATgD *in vitro* was examined to determine whether it could be extended to *in vivo* studies. 6×10^7 pfu of AdCMVCATgD virus were injected into adult rat hearts in a volume of 50 μ l. A parallel injection of 10 μ g of the plasmid pAdCMVCATgD was

CAT antigen at all doses of virus. Three doses of viral input are shown. A,B= 6×10^6 ; C,D= 6×10^7 ; E,F= 2×10^9 . Photographs of tissue sections were taken under Differential Interference Contrast (DIC) microscopy). A,C,E; Bar = 1mm, B,D,F; Bar = .05mm. CAT positive cells are stained brown for peroxidase reaction. All sections are counterstained with hematoxylin. In many regions, virtually 100% of myocytes stain positive. Positive cells include both myocytes and nonmyocytes, although it appears that the proportion of myocytes infected exceeds that of non-myocytes. A substantial number of inflammatory cells were seen (See Figure 21(d) & 21(e)). The nature of this inflammatory response is currently under investigation but does not appear to correlate with the amount of introduced virus. The intensity of peroxidase staining appeared to increase with increasing viral dose. It appears that the lowest dose of virus (6×10^6) resulted in a lower intensity of CAT antigen/cell as well as reduced number of infected cells. At higher doses of virus, both an increased number of cells and an increased amount of CAT/cell were obtained.

Cardiac myocytes appear to be ideally suited for the use of adenovirus mediated gene transfer. Transient transfection of fetal cardiocytes under optimized conditions traditionally results in 10-20% of the cells being transfected. Adenovirus can infect virtually 100% of cells and does not require the use of damaging treatments such as electroporation which generally kills a large number of the cells in the culture. Clearly, fetal cardiocytes possess viral receptors in numbers do not present a limitation to use of adenovirus vectors in rat cardiocytes. With adenovirus infection, there is no apparent effect on cell viability or morphology at the pfu ratios tested here. In addition, adenovirus infections also provide an efficient means of gene transfer into adult cells which has not been possible using conventional transfection strategies (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387, which disclosure is hereby incorporated by reference). A recent report of adenovirus infection of adult rat cardiocytes (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387, which disclosure is hereby incorporated by reference) reported 90% infection at a dose of 10^3 pfu/cell. Based on the results of the present invention, it is not necessary to use such a high dose of virus. Because of the efficient CAT expression system, the viral dose

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obtained in accordance with the present invention and those of Lemarchand *et al.*, 1993, *Circ. Res.*, vol. 72, pp. 1132-1138, which disclosure is hereby incorporated by reference, demonstrate a rather transient pattern of expression. It may be that in order to generate long-term expression it will be necessary to introduce the virus into neonates, as has been suggested by Stratford-Perricaudet *et al.*, 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630, which disclosure is hereby incorporated by reference. Studies are currently underway to examine the effect of various routes of infection, tissue distribution and immune response to this virus *in vivo*. However, it is apparent that adenovirus mediated gene transfer in the heart is extremely efficient and should be a very useful tool for the introduction of genes into cardiac myocytes.

It should be understood, that the foregoing embodiments are provided for purpose of illustration only and, not limitation, and that all such modifications or changes which occur to persons skilled in the art are deemed to be within the spirit and scope of the present invention.

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8. The vector according to Claim 1, wherein said vector comprises the nucleotide sequence as substantially shown in Figure 22 (Seq.Id.No.1).

9. The vector according to Claim 1, wherein said vector further comprises a
5 separate site for insertion of a second transcription unit.

10. A method of producing a recombinant adenovirus expression vector for expression of a heterologous gene(s) and/or gene product(s) in a host cell capable of being infected by said adenovirus, comprising:

- 10 a) preparing the vector according to Claim 1;
b) co-transfecting said vector with an adenovirus-5 genome in 293 cells, under conditions which facilitate homologous recombination between said vector and adenovirus-5, thereby producing a recombinant adenovirus; and
c) isolating the recombinant adenovirus.

15

11. A recombinant adenovirus expression vector produced according to the method of Claim 10.

12. A host cell line or animal infected by the recombinant adenovirus
20 expression vector according to Claim 11.

13. A unicellular host transformed by the vector according to Claim 1.

14. A method for producing a selected protein, comprising, culturing a host
25 which has been infected with a recombinant adenovirus vector according to Claim 11.

15. A method for producing a selected protein, comprising culturing a transformed host which has been transformed with a vector according to Claim 1.

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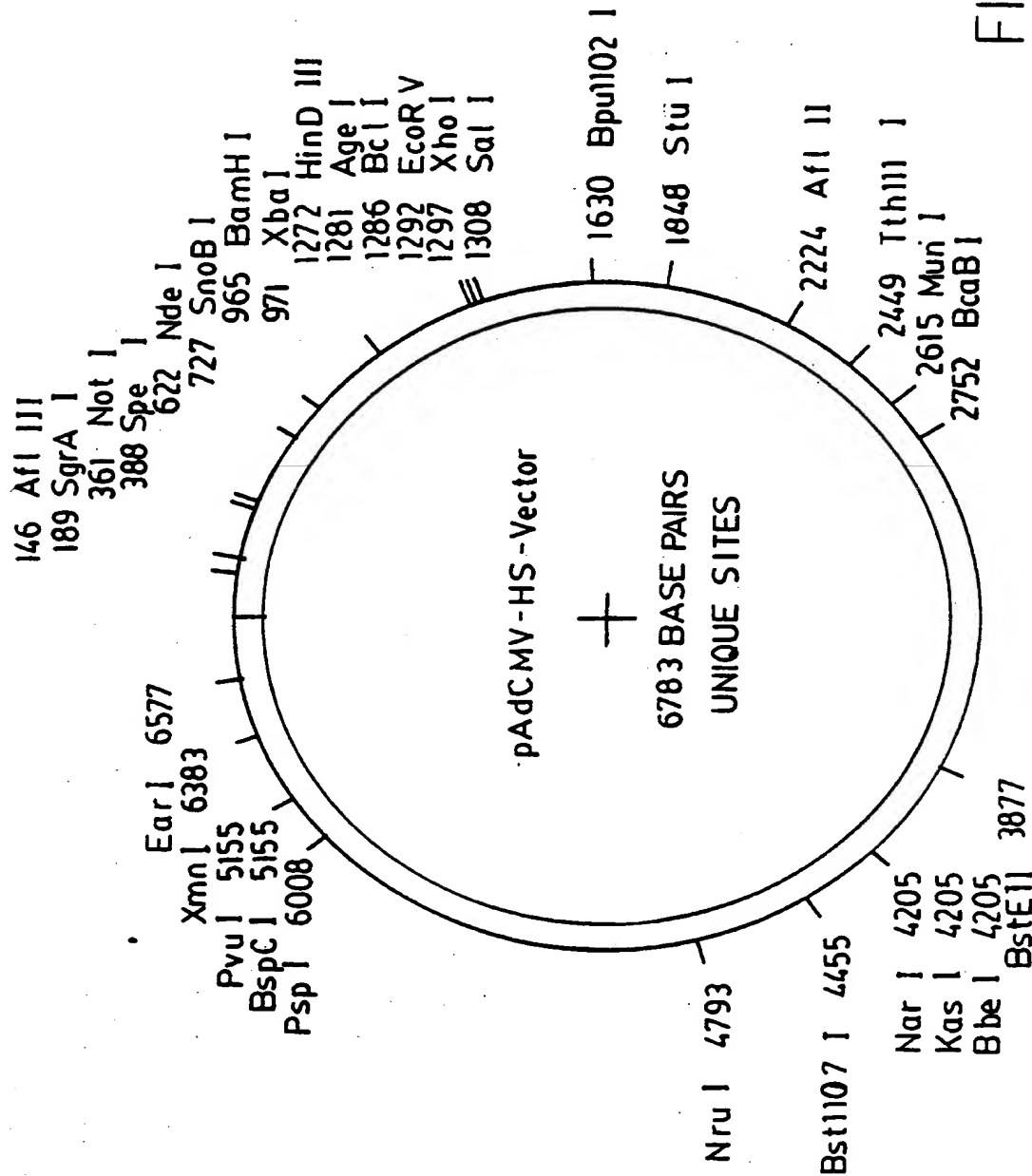
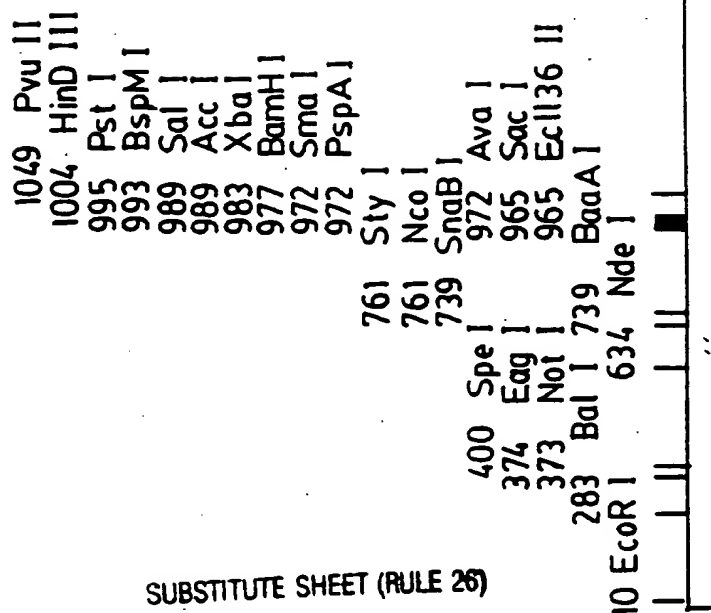


FIG. 1A

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FIG. 2



pGEM2AdCMV

3818 BASE PAIRS UNIQUE SITES

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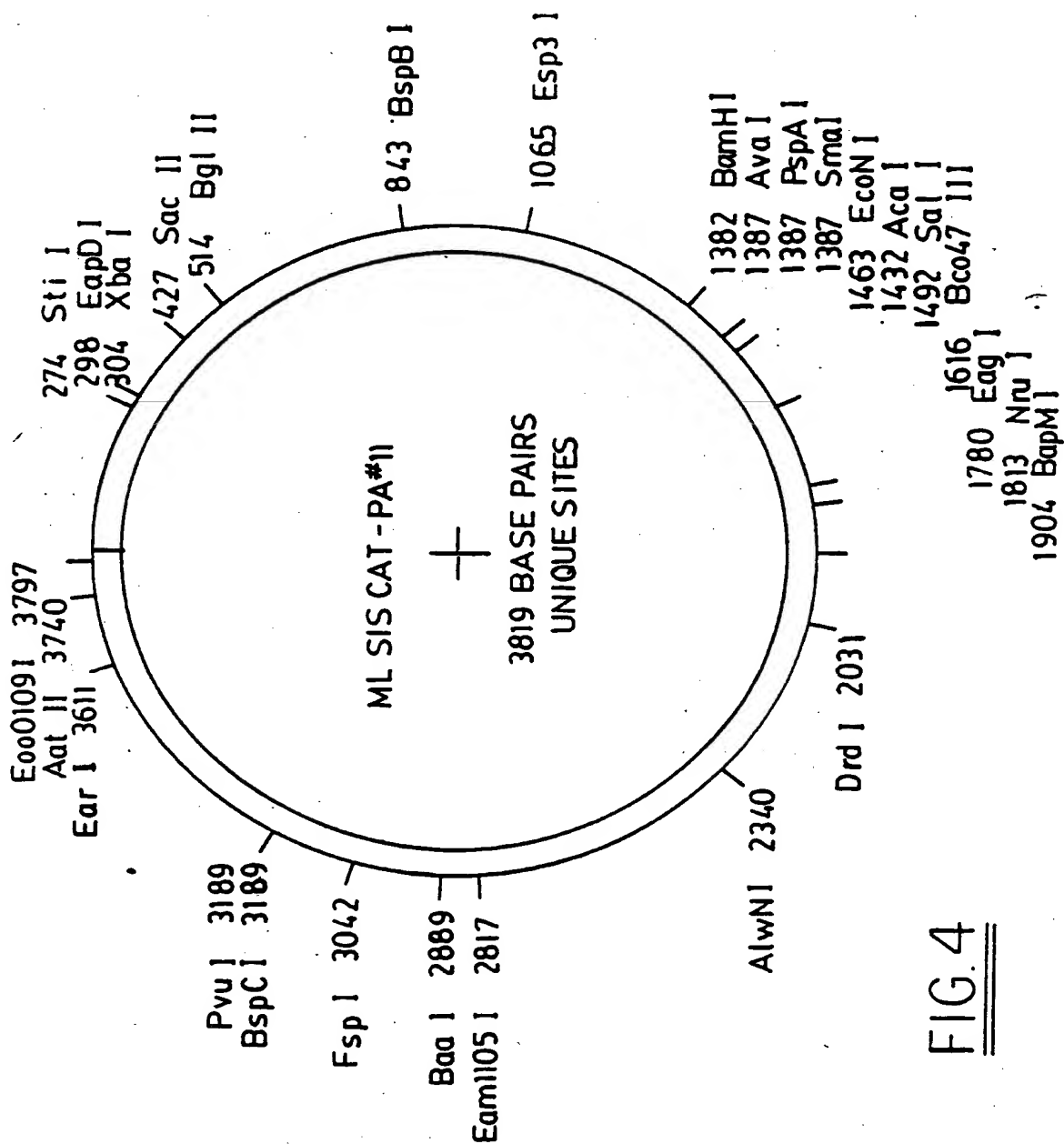


FIG. 4

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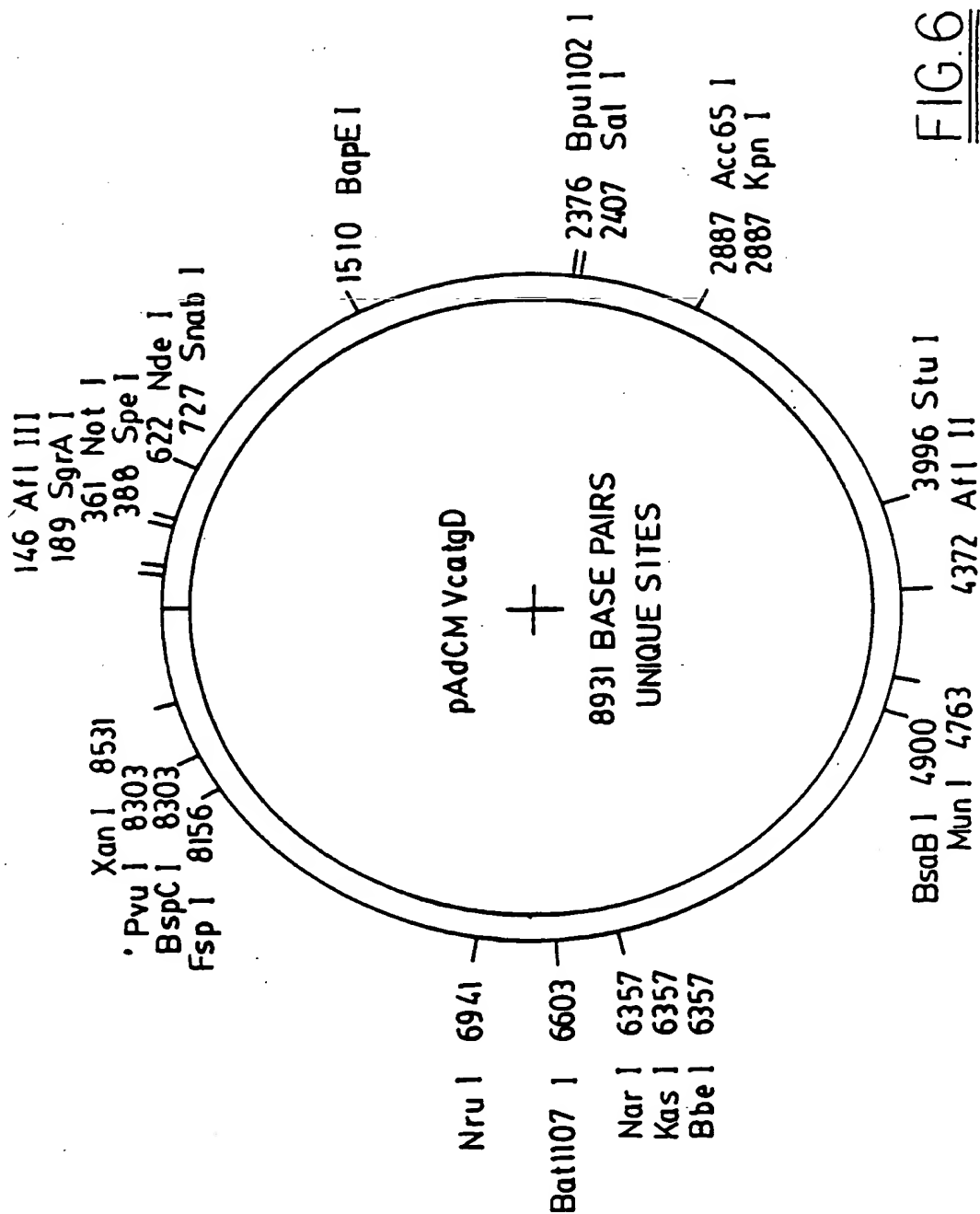


FIG. 6

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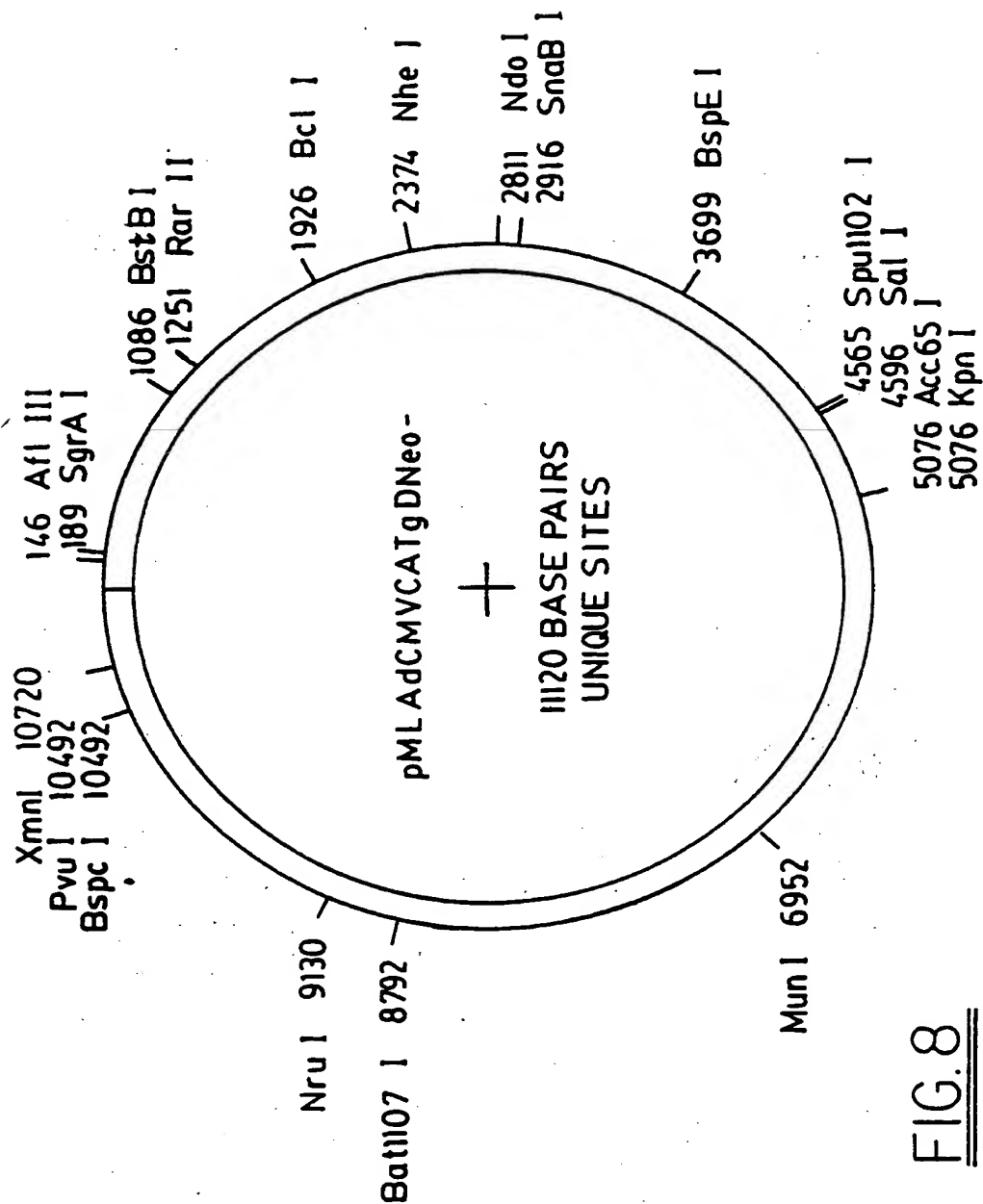


FIG. 8

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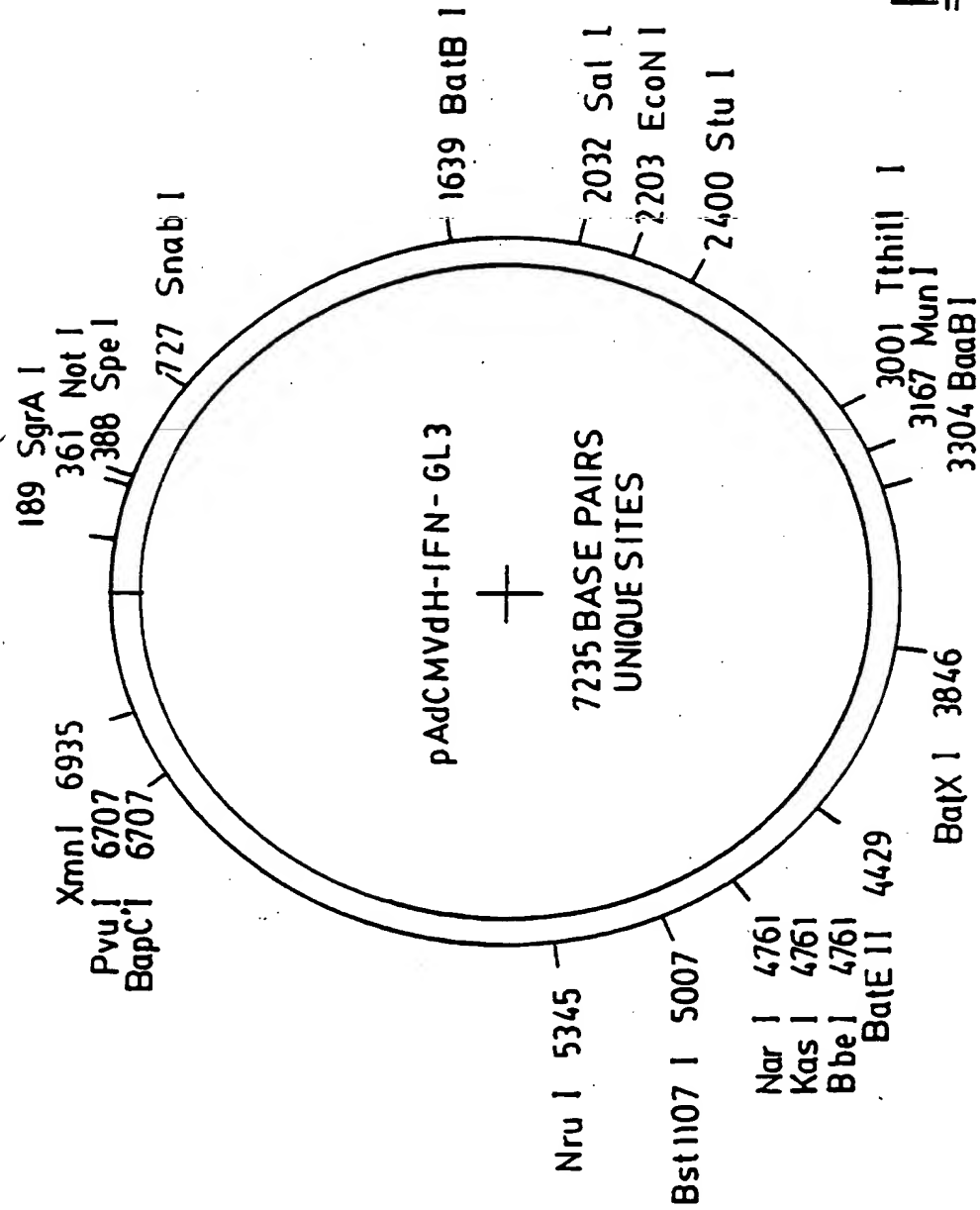


FIG. 10

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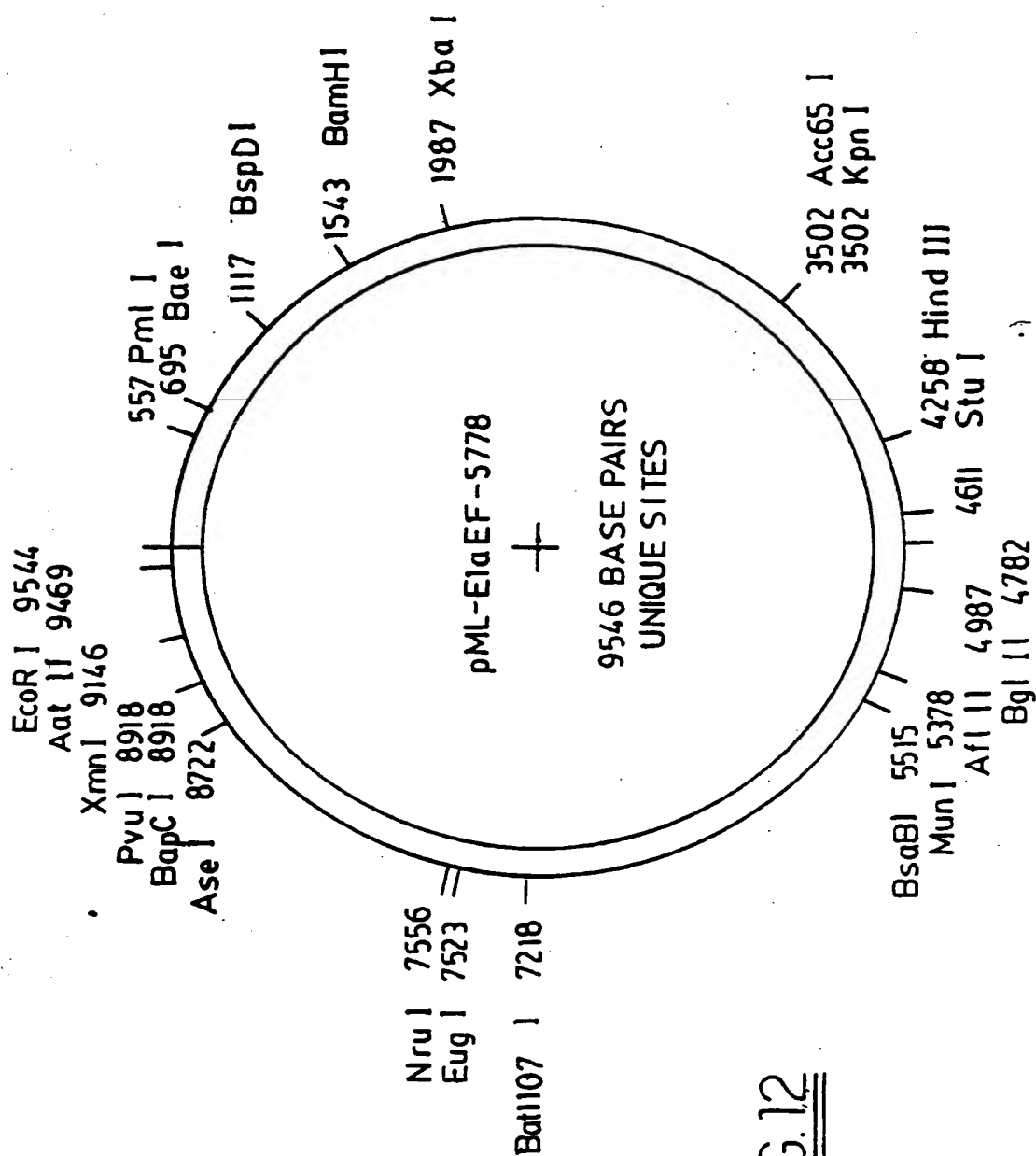
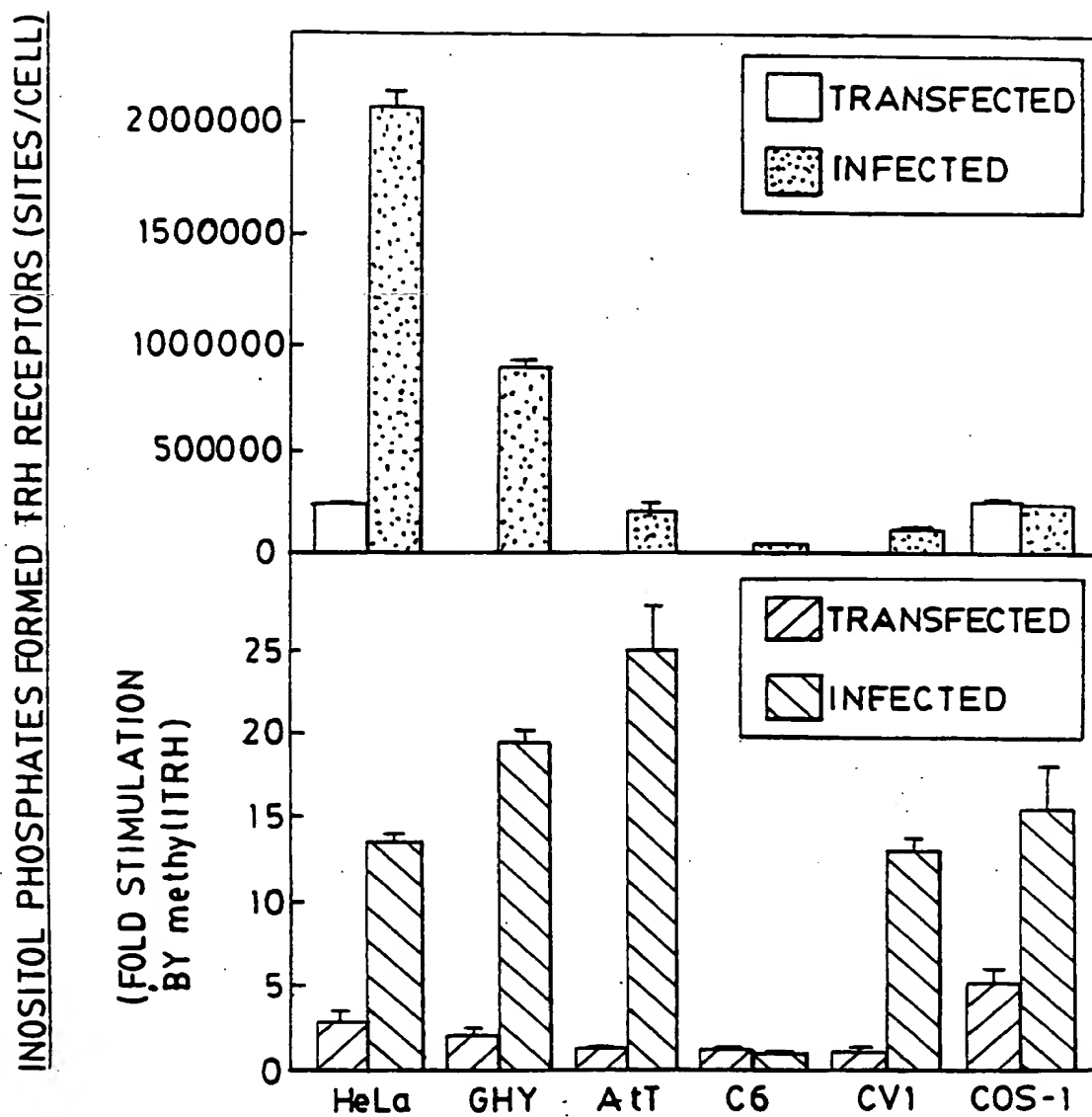
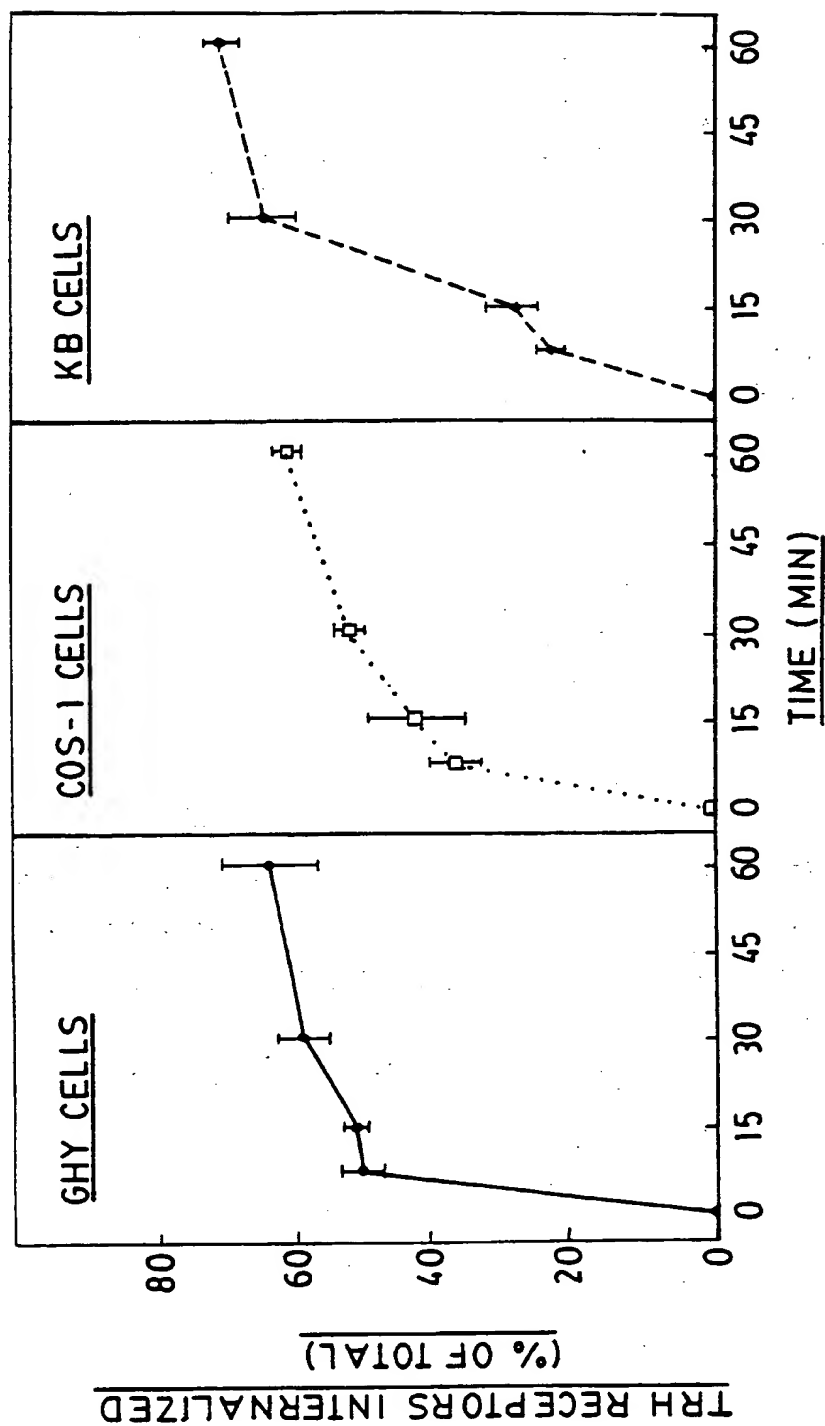


FIG. 12

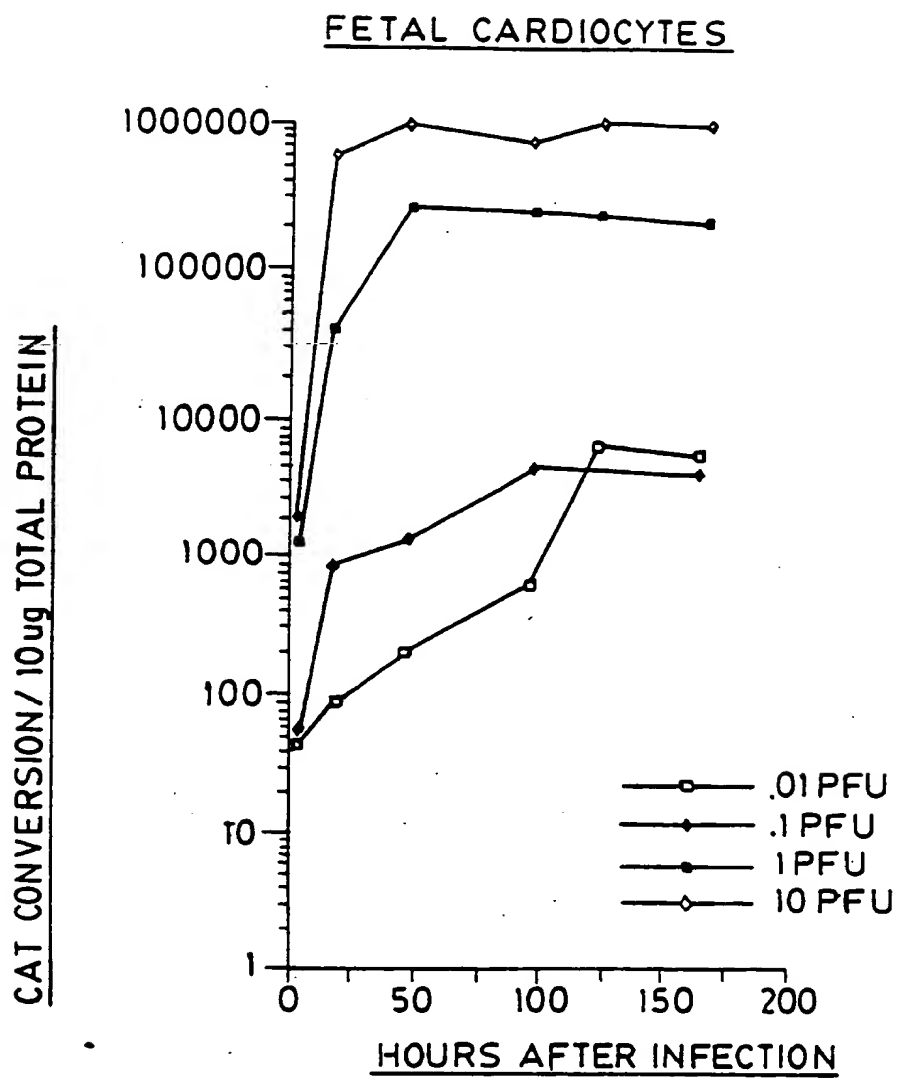
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FIG.14

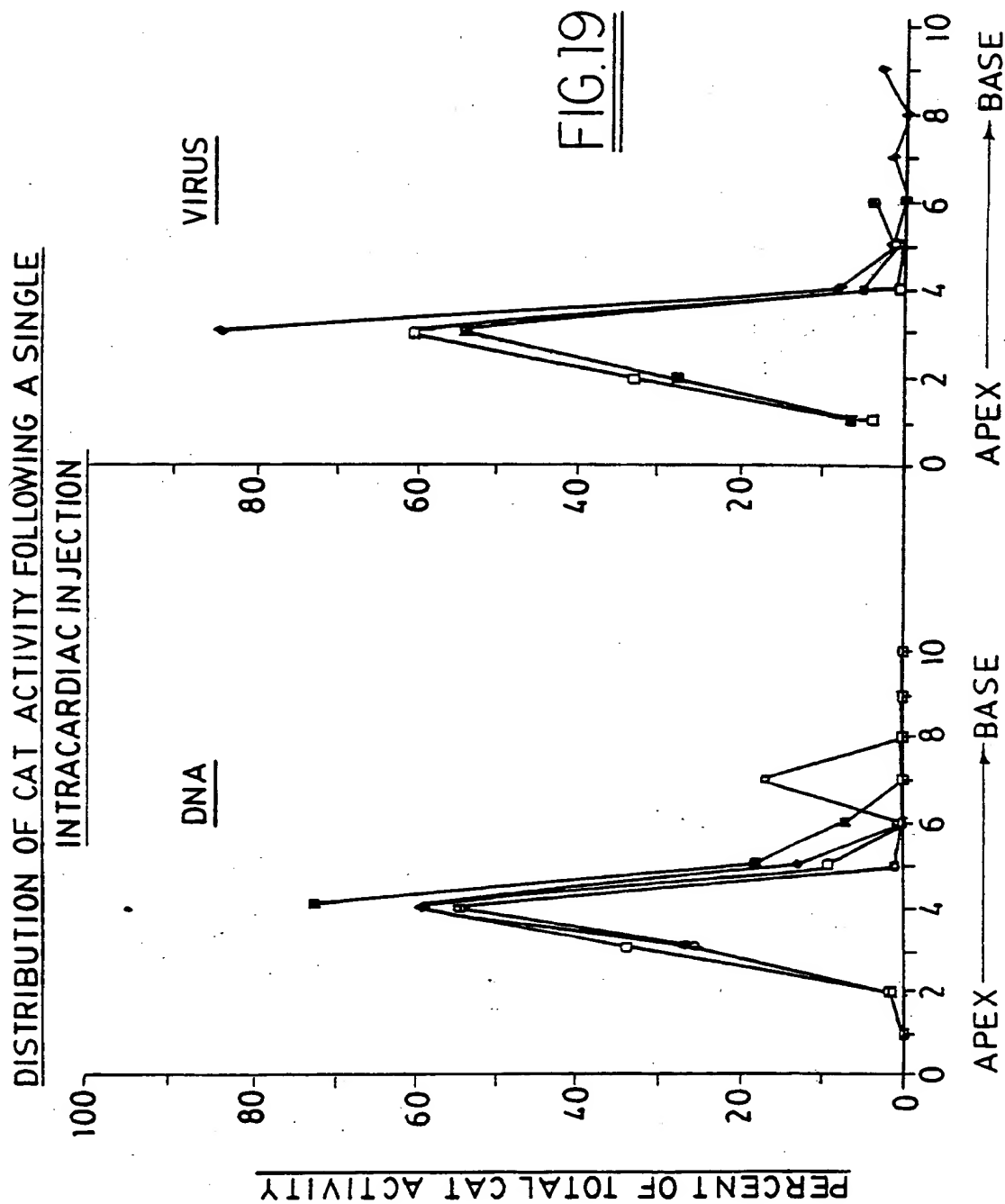
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FIG. 16

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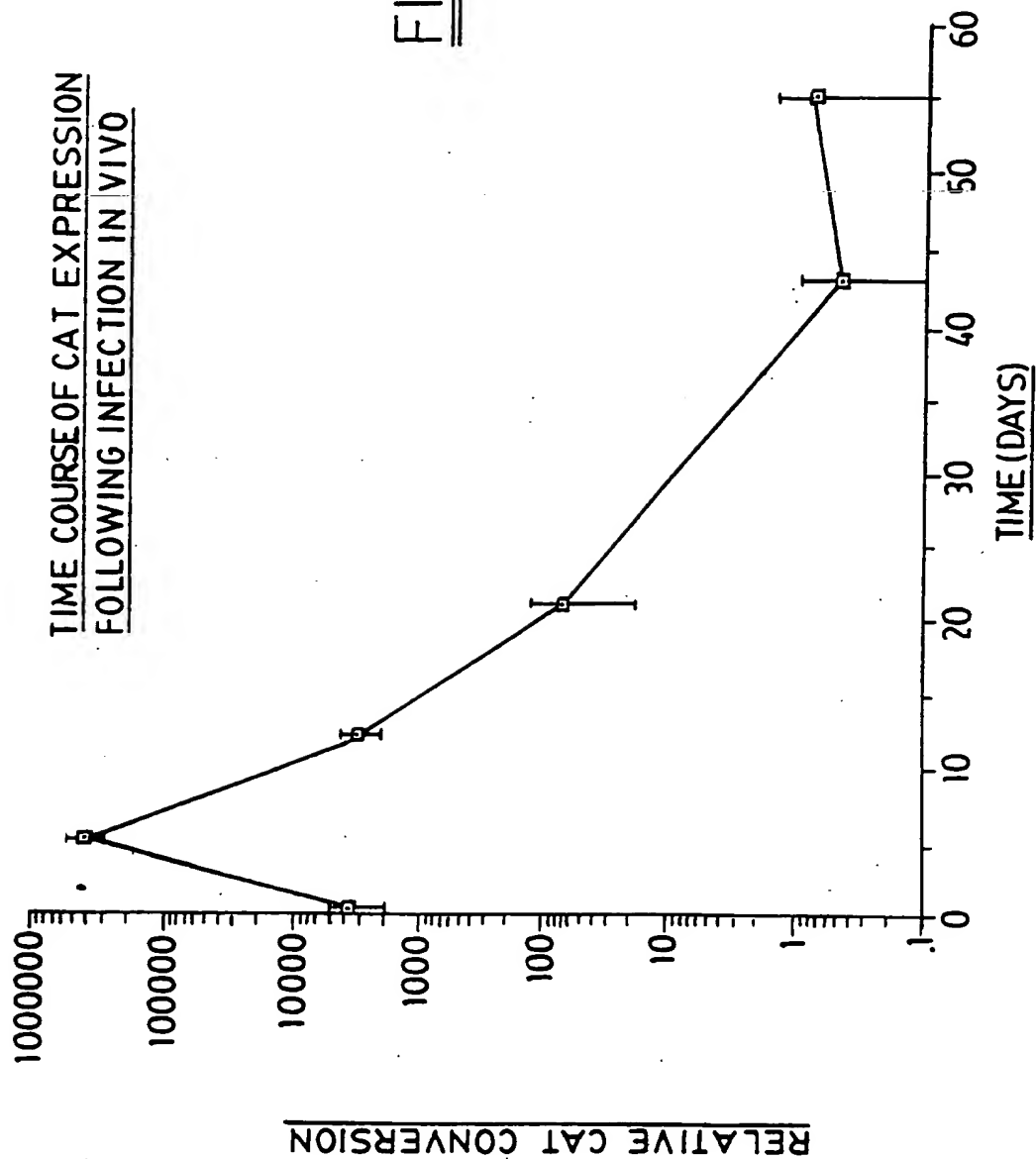
FIG. 18A

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FIG. 20B



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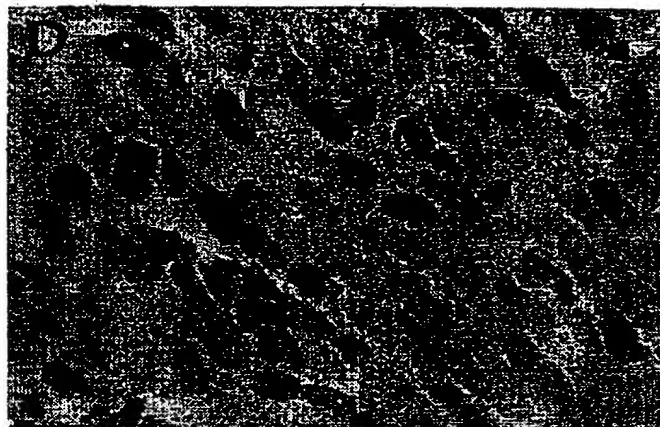


FIG. 21D

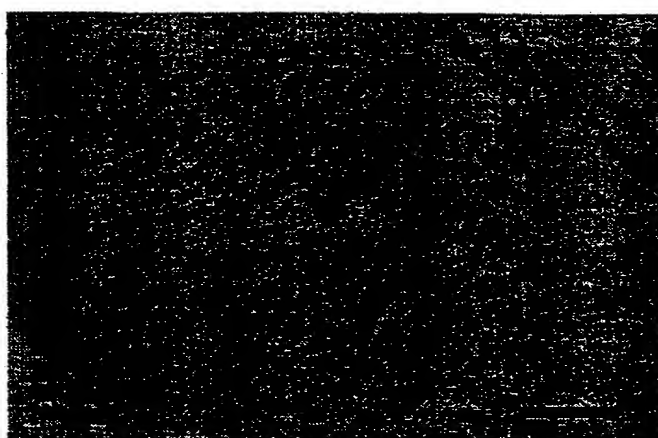


FIG. 21E



FIG. 21F

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Fig. 22 (continued)

ATATTCACCT GGCCCGCGGT GATGCCTTTG AGGGTGGCCG CGTCCATCTG
 GTCAGAAAAG 1140

ACAATCTTTT TGTGTGCAAA AGCGCTTGAG GTGTGGCAGG CTTGAGATCT
 GGCCATACAC 1200

TTGAGTGACA ATGACATCCA CTTTGCCTTT CTCTCCACAG GTGTCCACTC
 CCAGGTCCAA 1260

CTGCAGCCCC CAAGCTTGGG AATTCTCTCG GAAACGATGA AATATACAAG
 TTATATCTTG 1320

GCTTTTCAGC TCTGCATCGT TTTGGGTTCT CTTGGCTGTT ACTGCCAGGA
 CCCATATGTA 1380

AAAGAAGCAG AAAACCTTAA GAAATATTTT AATGCAGGTC ATTCAGATGT
 AGCGGATAAT 1440

GGAACTCTTT TCTTAGGCAT TTTGAAGAAT TGGAAAGAGG AGAGTGACAG
 AAAAATAATG 1500

CAGAGCCAAA TTGTCTCCTT TTA CTCTCAAA CTTTTTAAAA ACTTTAAAGA
 TGACCAGAGC 1560

ATCCAAAAGA GTGTGGAGAC CATCAAGGAA GACATGAATG TCAAGTTTTT
 CAATAGCAAC 1620

AAAAAGAAAC GAGATGACTT CGAAAAGCTG ACTAATTATT CGGTAAGTGA
 CTTGAATGTC 1680

CAACGCAAAG CAATACATGA ACTCATCCAA GTGATGGCTG AACTGTCGCC
 AGCAGCTAAA 1740

ACAGGGAAGC GAAAAAGGAG TCAGATGCTG TTTCAAGGTC GAAGAGCATC
 CCAGTAATGG 1800

TTGTCTCTGCG GATCCCTGCC AGTGGCGCAT AGCGATGCGC GGCAGAACCC
 CTTTGATTTT 1860

TAAACGGCGC AGACGGCAAG GGTGGGGGGT AAATAATCAC CCGAGAGTGT
 ACAAATAAAA 1920

ACATTTGCCT TTATTGAAAG TGTCTCCTAG TACATTATTT TTACATGTTT
 TTCAAGTGAC 1980

AAAAAGAAGT GGCGCTCCTA ATCTGCGCAC TGTGGCTGCG GGAGCTCTAG
 AGTCGACGGT 2040

ATCGCCCGAC ATCACCTGTG TCTATGGCCA CTGCCTTGGC TCACAAGTAC
 CACTAAACCC 2100

CCTTTCCTGC TCTTGCCTGT GAACAATGGT TAATTGTTCC CAAGAGAGCA
 TCTGTCAGTT 2160

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Fig. 22 (continued)

GCGGGATTGT GACTGACTTT GCTTTCCTGA GCCCGCTTGC AAGCAGTGCA
 GCTTCCCGTT 3300

CATCCGCCCCG CGATGACAAG TTGACGGCTC TTTTGGCACA ATTGGATTCT
 TTGACCCGGG 3360

AACTTAATGT CGTTTCTCAG CAGCTGTTGG ATCTGCGCCA GCAGGTTTCT
 GCCCTGAAGG 3420

CTTCCTCCCC TCCCAATGCG GTTTAAACA TAAATAAAAA ACCAGACTCT
 GTTTGGATTT 3480

GGATCAAGCA AGTGTCTTGC TGTCTTTATT TAGGGGTTTT GCGCGCGCGG
 TAGGCCCGGG 3540

ACCAGCGGTC TCGGTCGTTG AGGGTCCTGT GTATTTTTTC CAGGACGTGG
 TAAAGGTGAC 3600

TCTGGATGTT CAGATACATG GGCATAAGCC CGTCTCTGGG GTGGAGGTAG
 CACCACTGCA 3660

GAGCTTCATG CTGCGGGGTG GTGTTGTAGA TGATCCAGTC GTAGCAGGAG
 CGCTGGGCGT 3720

GGTGCCTAAA AATGTCTTTC AGTAGCAAGC TGATTGCCAG GGGCAGGCCC
 TTGGTGTAAG 3780

TGTTTACAAA GCGGTTAAGC TGGGATGGGT GCATACGTGG GGATATGAGA
 TGCATCTTGG 3840

ACTGTATTTT TAGGTTGGCT ATGTTCCCAG CCATATCCCT CCGGGGATTC
 ATGTTGTGCA 3900

GAACCACCAG CACAGTGTAT CCGGTGCACT TGGGAAATTT GTCATGTAGC
 TTAGAAGGAA 3960

ATGCGTGGA GAACTTGGAG ACGCCCTTGT GACCTCCAAG ATTTTCCATG
 CATTCTCCA 4020

TAATGATGGC AATGGGCCCA CGGGCGGCGG CCTGGGCGAA GATATTTCTG
 GGATCACTAA 4080

CGTCATAGTT GTGTTCCAGG ATGAGATCGT CATAGGCCAT TTTTACAAAG
 CGCGGGCGGA 4140

GGGTGCCAGA CTGCGGTATA ATGGTTCCAT CCGGCCCAGG GCGGTAGTTA
 CCCTCACAGA 4200

TTTGCATTTT CCACGCTTTG AGTTCAGATG GGGGGATCAT GTCTACCTGC
 GGGGCGATGA 4260

AGAAAACGGT TTCCGGGGTA GGGGAGATCA GCTGGGAAGA AAGCAGGTTC
 CTGAGCAGCT 4320

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Fig. 22 (continued)

CTTGACGCC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAAACGTT
TCGGCGAGAA 5460

GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGGC TACGTCTTGC
TGGCGTTCGC 5520

GACGCGAGGC TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCCGGCG
GCATCGGGAT 5580

GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG
GACAGETTCA 5640

AGGATCGCTC GCGGGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC
TCCGCCCCC 5700

TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA
CAGGACTATA 5760

AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC
CGACCCTGCC 5820

GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT
CTCAATGCTC 5880

ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT
GTGTGCACGA 5940

ACCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG
AGTCCAACCC 6000

GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA
GCAGAGCGAG 6060

GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT
ACACTAGAAG 6120

GACATATTTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA
GAGTTGGTAG 6180

CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTGTGTT
GCAAGCAGCA 6240

GATTACGCGC AGAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA
CGGGGTCTGA 6300

CGCTCAGTGG AACGAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT
CAAAAAGGAT 6360

CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTAA TCAATCTAAA
GTATATATGA 6420

GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT
CAGCGATCTG 6480

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Fig. 23

TTCCATCATC GAGGGGGTGG	AATAATATAC 60	CTTATTTTGG	ATTGAAGCCA	ATATGATAAT
AGTTTGTGAC GTGGCGGAAG	GTGGCGCGGG 120	GCGTGGAAC	GGGCGGGTG	ACGTAGTAGT
TGTGATGTTG AGTGACGTTT	CAAGTGTGGC 180	GGAACACATG	TAAGCGACGG	ATGTGGCAAA
TTGGTGTGCG GCGGATGTTG	CCGGTGTACA 240	CAGGAAGTGA	CAATTTTCGC	GCGGTTTTAG
TAGTAAATTT CTGAATAAGA	GGGCGTAACC 300	GAGTAAGATT	TGGCCATTTT	CGCGGGAAAA
GGAAGTGAAA CTAGGGCCTT	TCTGAATAAT 360	TTTGTGTTAC	TCATAGCGCG	TAATATTTGT
GCGGCCGCAA TACGGGGTCA	GTTGACATTG 420	ATTATTGACT	AGTTATTAAT	AGTAATCAAT
TTAGTTCATA TGGCCCGCCT	GCCCATATAT 480	GGAGTTCCGA	GTTACATAAC	TTACGGTAA
GGCTGACCGC TCCCATAGTA	CCAACGACCC 540	CCGCCCATTG	ACGTCAATAA	TGACGTATGT
ACGCGAATAG AACTGCCCCAC	GGACTTTCCA 600	TTGACGTCAA	TGGGTGGAGT	ATTTACGGTA
TTGGCAGTAC CAATGACGGT	ATCAAGTGTA 660	TCATATGCCA	AGTACGCCCC	CTATTGACGT
AAATGGCCCCG TACTTGGCAG	CCTGGCATTG 720	TGCCCAGTAC	ATGACCTTAT	GGGACTTTCC
TACATCTACG GTACATCAAT	TATTAGTCAT 780	CGCTATTACC	ATGGTGATGC	GGTTTTGGCA
GGGCGTGGAT TGACGTCAAT	AGCGTTTTGA 840	CTCACGGGGA	TTTCCAAGTC	TCCACCCCAT
GGGAGTTTGT CAACTCCGCC	TTTGGCACCA 900	AAATCAACGG	GACTTTCCAA	AATGTCGTAA
CCATTGACGC CAGAGCTCGC	AAAGGGTCGG 960	TAGGCGTGTA	CGGTGGGAGG	TCTATATAAG
CCGGGGATCC GTACTCCCTC	TCTAGAATTC 1020	GCTGTCTGCG	AGGGCCAGCT	GTTGGGGTGA

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Fig. 23 (continued)

CGATGAGACC	CGCACCAGGT	GCAGACCCTG	CGAGTGTGGC	GGTAAACATA
TTAGGAACCA	2100			
GCCTGTGATG	CTGGATGTGA	CCGAGGAGCT	GAGGCCCGAT	CACTTGGTGC
TGGCCTGCAC	2160			
CCGCGCTGAG	TTTGGCTCTA	GCTATGAAGA	TACAGATTGA	GGTACTGAAA
TGTGTGGGCG	2220			
TGGCTTAAGG	GTGGGAAAGA	ATATATAAGG	TGGGGGTCTT	ATGTAGTTTT
GTATCTGTTT	2280			
TGCAGCAGCC	GCCGCCGCCA	TGAGCACCAA	CTCGTTTGAT	GGAAGCATTG
TGAGCTCATA	2340			
TTTGACAACG	CGCATGCCCC	CATGGGCCGG	GGTGCGTCAG	AATGTGATGG
GCTCCAGCAT	2400			
TGATGGTCGC	CCCGTCCTGC	CCGCAAATC	TACTACCTTG	ACCTACGAGA
CCGTGTCTGG	2460			
AACGCCGTTG	GAGACTGCAG	CCTCCGCCGC	CGCTTCAGCC	GCTGCAGCCA
CCGCCCGCGG	2520			
GATTGTGACT	GACTTTGCTT	TCCTGAGCCC	GCTTGCAAGC	AGTGCAGCTT
CCCGTTTCATC	2580			
CGCCCGCGAT	GACAAGTTGA	CGGCTCTTTT	GGCACAATTG	GATTCTTTGA
CCCGGGAAC	2640			
TAATGTCGTT	TCTCAGCAGC	TGTTGGATCT	GCGCCAGCAG	GTTTCTGCCC
TGAAGGCTTC	2700			
CTCCCCTCCC	AATGCGGTTT	AAAACATAAA	TAAAAAACCA	GACTCTGTTT
GGATTTGGAT	2760			
CAAGCAAGTG	TCTTGCTGTC	TTTATTTAGG	GGTTTTGCGC	GCGCGGTAGG
CCCGGGACCA	2820			
GCGGTCTCGG	TCGTTGAGGG	TCCTGTGTAT	TTTTTCCAGG	ACGTGGTAAA
GGTGACTCTG	2880			
GATGTTCAGA	TACATGGGCA	TAAGCCCGTC	TCTGGGGTGG	AGGTAGCACC
ACTGCAGAGC	2940			
TTCATGCTGC	GGGGTGGTGT	TGTAGATGAT	CCAGTCGTAG	CAGGAGCGCT
GGGCGTGGTG	3000			
CCTAAAAATG	TCTTTCAGTA	GCAAGCTGAT	TGCCAGGGGC	AGGCCCTTGG
TGTAAGTGTT	3060			

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Fig. 23 (continued)

CTGGTCCTGC	TGGTGCTGAA	GCGCTGCCGG	TCTTCGCCCT	GCGCGTCGGC
CAGGTAGCAT	4140			
TTGACCATGG	TGTCATAGTC	CAGCCCCTCC	GCGGCGTGCG	CCTTGGCGCG
CAGCTTGCCC	4200			
TTGGAGGAGG	CGCCGCACGA	GGGGCAGTGC	AGACTTTTGA	GGGCGTAGAG
CTTGGGCGCG	4260			
AGAAATACCG	ATTCCGGGGA	GTAGGCATCC	GCGCCGCAGG	CCCCGCAGAC
GGTCTCGCAT	4320			
TCCACGAGCC	AGGTGAGCTC	TGGCCGTTTC	GGGTCAAAAA	CCAGGTTTCC
CCCATGCTTT	4380			
TTGATGCGTT	TCTTACCTCT	GGTTTCCATG	AGCCGGTGTC	CACGCTCGGT
GACGAAAAGG	4440			
CTGTCCGTGT	CCCCGTATAC	AGACTTGAGA	GGTCGAGCGA	TGCCCTTGAG
AGCCTTCAAC	4500			
CCAGTCAGCT	CCTTCCGGTG	GGCGCGGGGC	ATGACTATCG	TCGCCGCACT
TATGACTGTC	4560			
TTCTTTATCA	TGCAACTCGT	AGGACAGGTG	CCGGCAGCGC	TCTGGGTCAT
TTTCGGCGAG	4620			
GACCGCTTTC	GCTGGAGCGC	GACGATGATC	GGCCTGTTCG	TTGCGGTATT
CGGAATCTTG	4680			
CACGCCCTCG	CTCAAGCCTT	CGTCACTGGT	CCCGCCACCA	AACGTTTCGG
CGAGAAGCAG	4740			
GCCATTATCG	CCGGCATGGC	GGCCGACCGG	CTGGGCTACG	TCTTGCTGGC
GTTTCGCGACG	4800			
CGAGGCTGGA	TGGCCTTCCC	CATTATGATT	CTTCTCGCTT	CCGGCGGCAT
CGGGATGCCC	4860			
GCGTTGCAGG	CCATGCTGTC	CAGGCAGGTA	GATGACGACC	ATCAGGGACA
GCTTCAAGGA	4920			
TCGCTCGCGG	GTAAAAAGGC	CGCGTTGCTG	GCGTTTTTCC	ATAGGCTCCG
CCCCCCTGAC	4980			
GAGCATCACA	AAAATCGACG	CTCAAGTCAG	AGGTGGCGAA	ACCCGACAGG
ACTATAAAGA	5040			
TACCAGGCGT	TTCCCCCTGG	AAGCTCCCTC	GTGCGCTCTC	CTGTTCCGAG
CCTGCCGCTT	5100			

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Fig. 23 (continued)

TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG
TAAGTTGGCC 6180

GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT
CATGCCATCC 6240

GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA
ATAGTGTATG 6300

CGGCGACCGA GTTGCTCTTG CCCGGCGTCA ACACGGGATA ATACCGCGCC
ACATAGCAGA 6360

ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACTCTC
AAGGATCTTA 6420

CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGAC CCAACTGATC
TTCAGCATCT 6480

TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC
CGCAAAAAAG 6540

GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTCA
ATATTATTGA 6600

AGCATTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT
TTAGAAAAAT 6660

AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT
CTAAGAAACC 6720

ATTATTATCA TGACATTAACT CTATTAAAAT AGGCGTATCA CGAGGCCCTT
TCGTCTTCAA 6780

GAA

6783

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14502

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Clin. Invest., Volume 92, issued July 1993, L. A. Kirshenbaum et al., "Highly Efficient Gene Transfer into Adult Ventricular Myocytes by Recombinant Adenovirus," pages 381-387, see entire article.	1-16
Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, B. Quantin et al., "Adenovirus as an expression vector in muscle cells in vivo," pages 2581-2584, see entire article.	1-16
P,X	Proc. Natl. Acad. Sci. USA, Volume 90, issued 15 December 1993, A. Kass-Eisler et al., "Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes in vitro and in vivo," pages 11498-11502, see entire article.	1-16